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(54) BIOSYNTHETIC OSTEOGENIC PROTEINS AND OSTEOGENIC DEVICES CONTAINING THEM

BIOSYNTHETISCHE OSTEOGENE PROTEINE UND SIE ENTHALTENDE OSTEOGENE VORRICHTUNGEN

PROTEINES OSTEOGENIQUES BIOSYNTHETIQUES ET DISPOSITIFS OSTEOGENIQUES LES CONTENANT

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Description

This invention relates to osteogenic devices, to synthetic genes encoding proteins which can induce osteogenesis in mammals and methods for their production using recombinant DNA techniques and to synthetic forms of osteogenic protein.

Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in-the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo.

This putative bone inductive protein has been shown to have a molecular mass of less than 50 kilodaltons (kD). Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

The potential utility of these proteins has been widely recognized. It is contemplated that the availability of the pure protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (Proc. Natl. Acad. Sci. USA (1987) 80). Urist et al. (Proc. Soc. Exp. Biol. Med. (1984) 173:194-199) disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

Urist et al. (Proc. Natl. Acad. Sci. USA (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative bone inductive factors produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and apparently expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated. See also Urist et al., EP 0,212,474 entitled Bone Morphogenic Agents.

Wang et al. (Proc. Nat. Acad. Sci. USA (1988) 85: 9484-9488) discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

Wozney et al. (Science (1988) 242: 1528-1534) discloses the isolation of full-length cDNA's encoding the human equivalents of three polypeptides originally purified from bovine bone. The authors report that each of the three recombinantly expressed human proteins are independently or in combination capable of inducing cartilage formation. No evidence of bone formation is reported.

Summary of the Invention

This invention relates to the subject matter of the claims. It involves osteogenic devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation. Suitably modified as disclosed herein. The devices also may induce cartilage as well as bone formation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, containing dispersed osteogenic protein in the form of a biosynthetic construct.

Key to these developments was the successful preparation of substantially pure osteogenic protein by purification from bone, the elucidation of amino acid sequence and structure data of the native osteogenic protein, and insights involving study of the DNA and amino acid sequences of the natural source product. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from mammalian bone. Investigation of the properties and structure of the native form osteogenic protein then permitted the inventors to develop a rational design for non-native forms, i.e., forms never before known in nature, capable of inducing bone formation. As far as applicants are aware, the constructs disclosed herein constitute the first instance of the design of a functional, active protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

A series of consensus DNA sequences were designed with the goal of producing an active osteogenic protein. The sequences were based on partial amino acid sequence data obtained from the naturally sourced product and on observed homologies with unrelated genes reported in the literature, or the sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in procaryotes, purified, cleaved, refolded, combined with a matrix, implanted in an established animal model, and shown to have endochondral bone-inducing activity. The currently preferred active proteins comprise sequences designated COP5, COP7, COP16, and OP1. The amino acid sequences of these proteins are set forth below.

```
                1       10          20          30          40
COP5                 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
                           50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
COP7                 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                           50          60          70
HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR

                                          -10
                                       PKHHSQRARKKNKN
                1       10          20          30          40
COP16    CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
                           50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

```
                                                          -5
                                                         HQRQA
                  1        10         20         30          40
OP1               CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                              50         60         70
                  YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
                      80         90         100
                  ISVLYFDDSSNVILKKYRNMVVRACGCH
```

In these sequences and all other amino acid sequences disclosed herein, the dashes (-) are used as fillers only to line up comparable sequences in related proteins, and have no other function. Thus, amino acids 45-50 of COP7, for example, are NHAVV. Also, the numbering of amino acids is selected solely for purposes of facilitating comparisons between sequences. Thus, for example, the DF residues numbered at 9 and 10 of COP5 and COP7 may comprise residues, e.g., 35 and 36, of an osteogenic protein embodying the invention. Various leader or trailer sequences may be attached to the operative active region provided the osteogenic or chondrogenic activity of the protein is not destroyed.

Thus, in one aspect, the invention comprises a protein comprising an amino acid sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that it is capable of inducing endochondral bone formation when properly folded and implanted in a mammal in association with a matrix. Some of these sequences induce cartilage, but not bone. Also, the bone forming materials may be used to produce cartilage if implanted in an avascular locus, or if an inhibitor to full bone development is implanted together with the active protein. Thus, in another aspect, the invention comprises a protein less than about 200 amino acids long (for each chain) including a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that it is capable at least of cartilage formation when properly folded and implanted in a mammal in association with a matrix. The phrase "sufficiently duplicative", as used herein, is used to describe proteins having a degree of homology with the specific sequences disclosed herein and other, different amino acids but which nevertheless exhibit osteogenic or chondrogenic activity.

In one preferred aspect, these proteins comprise species of the generic amino acid sequences:

```
 1        10         20         30         40          50
      LXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX
         60         70         80         90         100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX
```

or

```
 1        10         20         30         40          50
CXXXXLXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX
         60         70         80         90         100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXXVXLXXYXXMXVXXCXCX
```

where the letters indicate the amino acid residues of standard single letter code, and the Xs each represent any one of the 22 naturally occurring amino acid residues. Preferred amino acid sequences within the foregoing generic sequences are:

```
 1        10         20         30         40          50
     LYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
      K S S L  QE VIS E FD Y  E A AY MPESMKAS   VI
      F E K I  DN     L    N  S   Q  ITK F P    TL
         A     S      K
        60         70         80         90         100
QTLVNSVNPGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP  A  EQMNSLAI FFNDQDK I RK EE T DA H H
    RF    T   S     K DPV V  Y N S     H RN   RS
     N    S                      K       P    E
```

and

```
 1        10         20         30         40         50
CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 RRRS K S S L  QE VIS E FD Y  E A AY MPESMKAS   VI
   KE F E K I  DN     L    N  S   Q  ITK F P    TL
    Q     A    S      K
         60         70         80         90         100
QTLVNSVNPGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP  A  EQMNSLAI FFNDQDK I RK EE T DA H H
    RF    T   S     K DPV V  Y N S     H RN   RS
     N    S                      K       P     E
```

wherein each of the amino acids arranged vertically at each position in the sequence may be used alternatively in various combinations. Note that these generic sequences have 6 and preferably 7 cysteine residues where inter- or intramolecular disulfide bonds can form, and contain other critical amino acids which influence the tertiary structure of the proteins. These generic structural features are found in previously published sequences, none of which have been described as capable of osteogenic activity, and most of which never have been linked with such activity.

Particular useful sequences include:

```
                1        10         20         30         40
Vg1             CKKRHLYVEFK-DVGWQNWVIAPQGYMANYCYGECPYPLTE
                          50         60         70
                ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
                    80         90         100
                ISMLFYDNNDNVVLRHYENMAVDECGCR

                1        10         20         30         40
DPP             CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
                          50         60         70
                HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
                    80         90         100
                VAMLYLNDQSTVVLKNYQEMTVVGCGCR

                 1        10         20         30         40
 CBMP-2a         CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                           50         60         70
                 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
                     80         90         100
                 ISMLYLDENEKVVLKNYQDMVVEGCGCR

                1        10         20         30         40
CBMP-2b         CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
                          50         60         70
                HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
                    80         90         100
                ISMLYLDEYDKVVLKNYQEMVVEGCGCR
```

```
                1       10          20          30          40
CBMP-3          CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
                          50          60          70
                SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
                    80          90          100
                LSILFFDENKNVVLKVYPNMTVESCACR

                1       10          20          30          40
COP1                 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
                          50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                    80          90          100
                ISMLYLDENSTVVLKNYQEMTVVGCGCR

                1       10          20          30          40
COP3                 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
                          50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                    80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
COP4                 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
                          50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                    80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

Vgl is a known Xenopus sequence heretofore not associated with bone formation. DPP is an amino acid sequence encoded by a Drosophila gene responsible for development of the dorsoventral pattern. OP1 is a region of a natural sequence encoded by exons of a genomic DNA sequence retrieved by applicants. The CBMPs are amino acid sequences comprising subparts of mammalian proteins encoded by genomic DNAs and cDNAs retrieved by applicants. The COPs are totally biosynthetic protein sequences expressed by novel consensus gene constructs, designed using the criteria set forth herein, and not yet found in nature.

These proteins are believed to be dimers. They appear not to be active when reduced. Various combinations of species of the proteins may exist as heterodimers or homodimers. As far as applicants are aware, the COP5, COP7, COP16, and OP1 constructs constitute the first instances of the design of a bioactive protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

The invention thus provides synthetic osteogenic protein produced using recombinant DNA techniques. The protein may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native protein, no matter how derived. In view of this disclosure, skilled genetic engineers can design and synthesize genes which encode appropriate amino acid sequences, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active synthetic proteins comprising truncated analogs, muteins, fusion proteins, and other constructs mimicking the biological activity of the native forms and capable of inducing bone formation in mammals including humans.

The synthetic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles or porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850 µm, preferably 70 - 420 µm. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable

in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and particulate, deglycosglated (or HF treated), protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin. Other useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures, and in other clinical applications including periodontal applications where bone formation is required.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 is a comparison of the amino acid sequence of various osteogenic proteins to those of the TGF-beta family. COP1, COP3, COP4, COP5, and COP7 are a family of analogs of synthetic osteogenic proteins developed from the consensus gene that was joined to a leader protein via a hinge region having the sequence D-P-N-G that permitted chemical cleavage at the D-P site (by acid) or N-G (by hydroxylamine) resulting in the release of the analog protein; VGI is a Xenopus protein, DPP is a Drosophila protein; OP1 is a native osteogenic protein; CBMP2a and 2b, and CBMP3 are subparts of proteins disclosed in PCT application 087/01537; MIS is Mullerian inhibitory substance; and "consensus choices" represent various substitutions of amino acids that may be made at various positions in osteogenic proteins;

FIGURE 2A is an E. coli expression vector containing a gene of an osteogenic protein fused to a leader protein;

FIGURE 2B is the DNA sequence comprising a modified trp-LE leader, two Fb domains of protein A, an ASP-PRO cleavage site, and the COP5 sequence;

FIGURES 3A and 3B are photomicrographs of implants showing the histology (day 12) of COP5 active recombinant protein. A is a control (rat matrix alone, 25 mg). B is rat matrix plus COP5, showing +++ cartilage formation and ++ bone formation (see key infra). Similar results are achieved with COP7; and

FIGURE 4 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene for osteogenic protein (COPO).

Description

Purification protocols have been developed which enable isolation of the osteogenic protein present in crude protein extracts from mammalian bone. The isolation procedure enables the production of significant quantities of substantially pure osteogenic protein from any mammalian species, provided sufficient amounts of fresh bone from the species is available. The empirical development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP has been characterized significantly; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat have been studied; it has been shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts; and it may be used to induce formation of endochondral bone in orthopedic defects including non-union fractures. In its native form it is a glycosylated, dimeric protein. However, it is active in deglycosylated form. It has been partially sequenced.

Elucidation of the amino acid sequence of BOP enabled the construction of a consensus nucleic acid sequence designed as disclosed herein based on the sequence data, inferred codons for the sequences, and observation of partial homology with known genes.

These consensus sequences have been refined by comparison with the sequences present in certain regulatory genes from drosophila, xenopus, and human followed by point mutation, expression, and assay for activity. This approach has been successful in producing several active totally synthetic constructs not found in nature (as far as applicants are aware) which have true osteogenic activity.

These discoveries enable the construction of DNAs encoding totally novel, non-native protein constructs which

individually and combined are capable of producing true endochondral bone. The DNAs may be expressed using well established recombinant DNA technologies in procaryotic or eucaryotic host cells, and the expressed proteins may be oxidized and refolded in vitro if necessary for biological activity.

The design and production of such biosynthetic proteins, the nature of the matrix, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following, which constitutes the best method currently known for practicing the various aspects of the invention.

CONSENSUS SEQUENCE DESIGN

A synthetic consensus gene shown in FIGURE 4 was designed to encode a consensus protein based on amino acid predictions from homology with the TGF-beta gene family. The designed concensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

Tryptic peptides derived from Bovine Osteogenic Protein isolated by applicants and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopus VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 1.

TABLE 1

protein	amino acid sequence	homology
(BOP)	SFDAYYCSGACQFPS ***** * * **	(9/15 matches)
(DPP)	GYDAYYCHGKCPFFL	
(BOP)	SFDAYYCSGACQFPS * ** * * *	(6/15 matches)
(Vg1)	GYMANYCYGECPYPL	
(BOP)	SFDAYYCSGACQFPS * ** * *	(5/15 matches)
(inhibin)	GYHANYCEGECPSHI	
(BOP)	SFDAYYCSGACQFPS * * * *	(4/15 matches)
(TGF-beta)	GYHANFCLGPCPYIW	
(BOP)	K/RACCVPTELSAISMLYLDEN ***** * **** * *	(12/20 matches)
(Vg1)	LPCCVPTKMSPISMLFYDNN	
(BOP)	K/RACCVPTELSAISMLYLDEN * ***** * **** *	(12/20 matches)
(inhibin)	KSCCVPTKLRPMSMLYYDDG	
(BOP)	K/RACCVPTELSAISMLYLDE **** * *	(6/19 matches)
(TGF-beta)	APCCVPQALEPLPIVYYVG	
(BOP)	K/RACCVPTELSAISMLYLDEN ******* * ****	(12/20 matches)
(DPP)	KACCVPTQLDSVAMLYLNDQ	

(BOP)	LYVDF	
	*****	(5/5 matches)
(DPP)	LYVDF	
(BOP)	LYVDF	
	*** *	(4/5 matches)
(Vgl)	LYVEF	
(BOP)	LYVDF	
	** **	(4/5 matches)
(TGF-beta)	LYIDF	
(BOP)	LYVDF	
	* *	(2/5 matches)
(inhibin)	FFVSF	

$\overline{*}$-match

In determining an appropriate amino acid sequence of an osteogenic protein (from which the nucleic acid sequence can be determined), the following points were considered: (1) the amino acid sequence determined by Edman degradation of natural source osteogenic protein tryptic fragments is ranked highest as long as it has a strong signal and shows homology or conservative changes when aligned with the other members of the gene family; (2) where the sequence matches for all four proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vgl are used; (4) If Vgl or DPP diverged but either one were matched by inhibin or by TGF-beta, this matched amino acid is chosen; (5) where all sequences diverged, the DPP sequence is initially chosen, with a later plan of creating the Vgl sequence by mutagenesis kept as a possibility. In addition, the consensus sequence is designed to preserve the disulfide crosslinking and the apparent structural homology among the related proteins.

RECOMBINANT OSTEOGENIC PROTEIN CONSTRUCTS

This approach resulted in the production of novel recombinant proteins capable of inducing formation of cartilage and endochondral bone comprising a protein structure analogous to or duplicative of the functional domain of the naturally sourced material. The amino acid sequences encoded by the consensus DNA sequences were derived from a family of natural proteins implicated in tissue development. These gene products/proteins are known to exist in active form as dimers and are, in general, processed from a precursor protein to produce an active C-terminal domain of the precursor.

The recombinant osteogenic/chondrogenic proteins are "novel" in the sense that, as far as applicants are aware, they do not exist in nature or, if they do exist, have never before been associated with bone or cartilage formation. The approach to design of these proteins is to employ amino acid sequences, found in the native OP isolates, in polypeptide structures are patterned after certain proteins reported in the literature, or the amino acid sequences inferred from DNAs reported in the literature. Thus, using the design criteria set forth above, and refining the amino acid sequence as more protein sequence information was learned, a series of synthetic proteins were designed with the hope and intent that they might have osteogenic or chondrogenic activity when tested in the bioassay system disclosed below.

It was noted, for example, that DPP from drosophila, VG1 from Xenopus, the TGF beta family of proteins, and to a lesser extent, alpha and beta inhibins, had significant homologies with certain of the sequences derived from the naturally sourced OP product. (FIGURE 1.) Study of these proteins led to the realization that a portion of the sequence

of each had a structural similarity observable by analysis of the positional relationship of cysteines and other amino acids which have an important influence on three dimensional protein conformation. It was noted that a region of these sequences had a series of seven cysteines, placed very nearly in the same relative positions, and certain other amino acids in sequence as set forth below:

```
          10         20         30         40         50
CXXXXLXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX
          60         70         80         90         100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid. Expression experiments of two of these constructs demonstrate activity. Expression experiments with constructs patterned after this template amino acid sequence with a shorter sequence having only six cysteines also show activity:

```
          10         20         30         40         50
     LXVXFXDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXNHAXX
          60         70         80         90         100
QXXVXXXNXXXXPXXCCXPXXXXXXXXLXXXXXXXVXLXXYXXMXVXXCXCX
```

wherein each X independently represents an amino acid. Within these generic structures are a multiplicity of specific sequences which have osteogenic or chondrogenic activity. Preferred structures are those having the amino acid sequence:

```
          10         20         30         40         50
CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 RKRS K S S L  QE VIS E FD Y  E A AY MPESMKAS   VI
   KE F E K I  DN     L    N  S   Q  ITK F P    TL
    Q     A    S      K
          60         70         80         90         100
QTLVNSVNPGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP  A EQMNSLAI FFNDQDK I RK EE T DA H H
    RF    T   S     K DPV V  Y N S     H RN   RS
     N    S                      K       P     E
```

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be used. Novel active proteins also are defined by amino acid sequences comprising an active domain beginning at residue number 6 of this sequence, i.e, omitting the N terminal CXXXX, or omitting any of the preferred specific combinations-such as CKRHP, CRRKQ, CKRHE, etc, resulting in a construct having only six cysteine residues. After this work, PCT 87/01537 was published, and it was observed that the proteins there identified as BMPII a and b and BMPIII each included a region embodying this generic structure. These proteins were not demonstrated to be osteogenic in the published application. However, applicants discovered that a subpart of the amino acid sequence of these proteins, properly folded, and implanted as set forth herein, is active. These are disclosed herein as CBMPIIa, CBMPIIb, and CBMPIII. Also, applicants retrieved a previously unreported gene by probing a human genomic DNA library with COPO. This protein was designated OP1. It comprises a region exhibiting the same generic structure.

Thus, the preferred osteogenic proteins are expressed from recombinant DNA and comprise amino acid sequences including any of the following sequences:

```
                1        10         20         30         40
     Vgl        CKKRHLYVEFK-DVGWQNWVIAPQGYMANYCYGECPYPLTE
                              50         60         70
                ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
                    80         90         100
                ISMLFYDNNDNVVLRHYENMAVDECGCR
```

```
                1       10          20          30          40
DPP             CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
                             50          60          70
                HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
                     80          90          100
                VAMLYLNDQSTVVLKNYQEMTVVGCGCR

                1       10          20          30          40
OP1                  LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                             50          60          70
                YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
                     80          90          100
                ISVLYFDDSSNVILKKYRNMVVRACGCH

                                                      -5
                                                      HQRQA
                1       10          20          30          40
OP1             CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                             50          60          70
                YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
                     80          90          100
                ISVLYFDDSSNVILKKYRNMVVRACGCH

                1       10          20          30          40
CBMP-2a         CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                             50          60          70
                HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQDMVVEGCGCR

                1       10          20          30          40
CBMP-2b         CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
                             50          60          70
                HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
                     80          90          100
                ISMLYLDEYDKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
CBMP-3          CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
                             50          60          70
                SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
                     80          90          100
                LSILFFDENKNVVLKVYPNMTVESCACR
```

```
                1       10          20          30          40
COP1                 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
                           50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                     80          90          100
                ISMLYLDENSTVVLKNYQEMTVVGCGCR

                1       10          20          30          40
COP3                 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
                           50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
COP4                 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
                           50          60          70
                HFNSTN--H-AVVQTLVNNMNPGK-VPKPCCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
COP5                 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
                           50          60          70
                HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR

                1       10          20          30          40
COP7                 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                           50          60          70
                HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR

                                              10
                                           PKHHSQRARKKNKN
                1       10          20          30          40
COP16           CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
                           50          60          70
                HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
                     80          90          100
                ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

As shown in FIGURE 1, these sequences have considerable homology with the alpha and beta inhibins, three forms of TGF beta, and MIS.

Gene Preparation

The synthetic genes designed to express the proteins as described above preferably are produced by assembly

of chemically synthesized oligonucleotides. 15-l00mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purifed by PAGE. Natural gene sequences and cDNAs also may be used for expression.

Expression

The genes can be expressed in appropriate prokaryotic hosts such as various strains of E. coli and also in bacillus, yeasts, and various animal cells such as CHO, myeloma, etc. Generally, expression may be achieved using many cell types and expression systems well known to those skilled in the art. If the gene is to be expressed in E. coli, it must first be cloned into an expression vector. An expression vector (FIGURE 2A) based on pBR322 and containing a synthetic trp promoter operator and the modified trp LE leader can be opened at the EcoRI and PSTI restriction sites, and a FB-FB COP1, COP3, COP5, and COP7 gene fragments (FIGURE 2B) can be inserted between these sites, where FB is fragment B of Staphylococcal Protein A. The expressed fusion protein results from attachment of the COP gene to a fragment encoding FB. The COP protein is joined to the leader protein via a hinge region having the sequence Asp-Pro-Asn-Gly. This hinge permits chemical cleavage of the fusion protein with dilute acid at the asp-pro site or cleavage at Asn-Gly with hydroxylamine, resulting in release of the COP protein. For COP16 and OP1, the proteins are expressed as fusion products, using the modified trp-LE as a leader.

Production of Active Proteins

The following procedure was followed for production of active recombinant proteins. E. coli cells containing the fusion proteins were lysed. The fusion proteins were purified by differential solubilization. In the case of the COP1, 3, 4, 5, and 7 fusion proteins, cleavage was with dilute acid, and the resulting cleavage products were passed through a Sephacryl-200HR column. The Sephacryl column separated most of the uncleaved fusion products from the COP1, 3, 4, 5, and 7 analogs. In the case of the COP16 or OP1 fusion protein, cleavage was with a more concentrated acid, and an SP-Trisacryl column was used as an additional purification step. The COP or OP fractions were then subjected to HPLC on a semi-prep C-18 column.

Initial conditions for refolding of COP analogs or OP1 were at pH 8.0 using Tris, Gu-HCl, dithiothreitol. Final conditions for refolding of COP analogs were at pH 8.0 using Tris, oxidized glutathione, and lower amounts of Gu-HCl and dithiothreitol. Alternatively, the COP or OP1 proteins are suspended in 50 mM HCl, 6 M guanidine-HCl, pH 8.0, for 18 hours at 4°C. Refolding may not be required if the proteins are expressed in animal cells.

Production of Antisera

Antisera to COP7 and COPS were produced in New Zealand white rabbits. Western blots demonstrate that the antisera react with COP7 and COP5 preparations. Antisera to COP7 has been tested for reactivity to naturally sourced bovine osteogenic protein samples. Western blots show a clear reaction with the 30 kD protein and, when reduced, with the 16 kD subunit. The immunoreactive species appears as a closely-spaced doublet in the 16 kD subunit region, similar to the 16 kD doublet seen in Con A blots.

MATRIX PREPARATION

General Consideration of Matrix Properties

The carrier described in the bioassay section, infra, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 μm elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate osteogenic protein onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

The sequential cellular reactions at the interface of the bone matrix/OP implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow

differentiation.

A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible in vivo and biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Biocompatibility requires that the matrix not induce significant inflammation when implanted and not be rejected by the host animal. Biodegradability requires that the matrix be slowly absorbed by the body of the host during development of new bone or cartilage. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

Matrix geometry, particle size, the presence of surface charge, and porosity or the presence of interstices among the particles of a size sufficient to permit cell infiltration, are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogeneic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles and the dispersed osteogenic protein.

Preparation of Biologically Active Allogenic Matrix

Demineralized bone matrix is prepared from the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which pass through a 420 μm sieve. The bone particles are subjected to dissociative extraction with 4 M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and false positives before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure protein is reconstituted with the biologically inactive insoluble collagenous matrix. The osteoinductive protein can be obtained from any vertebrate, e.g., bovine, porcine, monkey, or human, or produced using recombinant DNA techniques.

Preparation of Deglycosylated Bone Matrix for Use in Xenoaenic Implant

When osteogenic protein is reconstituted with collagenous bone matrix from other species and implanted in rat, no bone is formed. This suggests that while the osteogenic protein is xenogenic (not species specific), while the matrix is species specific and cannot be implanted cross species perhaps due to intrinsic immunogenic or inhibitory components. Thus, heretofore, for bone-based matrices, in order for the osteogenic protein to exhibit its full bone inducing activity, a species specific collagenous bone matrix was required.

The major component of all bone matrices is Type I collagen. In addition to collagen, extracted bone includes non-collagenous proteins which may account for 5% of its mass. Many non-collagenous components of bone matrix are glycoproteins. Although the biological significance of the glycoproteins in bone formation is not known, they may present themselves as potent antigens by virtue of their carbohydrate content and may constitute immunogenic and/or inhibitory components that are present in xenogenic matrix.

It has now been discovered that a collagenous bone matrix may be used as a carrier to effect bone inducing activity in xenogenic implants, if one first removes the immunogenic and inhibitory components from the matrix. The matrix is deglycosglated chemically using, for example, hydrogen fluoride to achieve this purpose.

Bovine bone residue prepared as described above is sieved, and particles of the 74-420 μM are collected. The sample is dried in vacuo over P_2O_5, transferred to the reaction vessel and anhydrous hydrogen fluoride (HF) (10-20 ml/g of matrix) is then distilled onto the sample at -70°C. The vessel is allowed to warm to 0°C. and the reaction mixture is stirred at this temperature for 120 min. After evaporation of the HF in vaccuo, the residue is dried thoroughly in vaccuo over KOH pellets to remove any remaining traces of acid.

Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with HF, after washing the samples appropriately to remove non-covalently bound carbohydrates.

The deglycosylated bone matrix is next treated as set forth below:

1) suspend in TBS (Tris-buffered Saline) lg/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) and stir at RT for 30 min;

2) centrifuge and wash with TBS or UTBS as in step 1; and

3) centrifuge; discard supernatant; water wash residue; and then lyophilize.

FABRICATION OF DEVICE

Fabrication of osteogenic devices using any of the matrices set forth above with any of the osteogenic proteins described above may be performed as follows.

A. Ethanol precipitation

In this procedure, matrix is added to osteogenic protein in guanidine-HCl. Samples are vortexed and incubated at a low temperature. Samples are then further vortexed. Cold absolute ethanol is added to the mixture which is then stirred and incubated. After centrifugation (microfuge high speed) the supernatant is discarded. The reconstituted matrix is washed with cold concentrated ethanol in water and then lyophilized.

B. Acetonitrile Trifluoroacetic Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluroacetic acid (ACN/TFA) solution is added to the carrier. Samples are vigorously vortexed many times and then lyophilized.

C. Urea Lyophilization

For those proteins that are prepared in urea buffer, the protein is mixed with the matrix, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

IN VIVO RAT BIOASSAY

Several of the synthetic proteins have been incorporated in matrices to produce osteogenic devices, and assayed in rat for endochondral bone. Studies in rats show the osteogenic effect to be dependent on the dose of osteogenic protein dispersed in the osteogenic device. No activity is observed if the matrix is implanted alone. The following sets forth guidelines for how the osteogenic devices disclosed herein can be assayed for evaluating protein constructs and matrices for biological activity.

A. Subcutaneous Implantation

The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, is used to assess endochondral bone differentiation activity. This assay consists of implanting the test samples in subcutaneous sites in allogenic recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoraic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The heterotropic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

B. Cellular Events

The implant model in rats exhibits a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartiliage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

C. Histological Evaluation

Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants

are fixed in Bouins Solution, embedded in parafilm, cut into 6-8 mm sections. Staining with toluidine blue or hemotoxylin/ eosin demonstrates clearly the ultimate development of endochondrial bone. Twelve day implants are usually sufficient to determine whether the implants show bone inducing activity.

D. Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology should have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. Alternatively the amount of bone formation can be determined by measuring the calcium content of the implant.

The osteogenic activity due to osteogenic protein is represented by "bone forming units". One bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

Devices that contained only rat carrier show complete absence of new bone formation. The implant consists of carrier rat matrix and surrounding mesenchymal cells. Again, the devices that contained rat carrier and not correctly folded (or biologically inactive) recombinant protein also showed complete absence of bone formation. These implants are scored as cartilage formation (-) and bone formation (-). The endochondral bone formation activity is scored as zero percent (0%) (FIGURE 3A).

Implants included biologically active recombinant protein, however, showed evidence of endochondral bone formation. Histologically they showed new cartilage and bone formation.

The cartilage formation is scored as (+) by the presence of metachromatically stained chondrocytes in the center of the implant, as (++) by the presence of numerous chondrocytes in many areas of the implant and as (+++) by the presence of abundant chondrocytes forming cartilage matrix and the appearance of hypertrophied chondrocytes accompanying cartilage calcification (FIGURE 3B).

The bone formation is scored as (+) by the presence of osteoblast surrounding vascular endothelium forming new matrix, as (++) by the formation of bone due to osteoblasts (as indicated by arrows) and further bone remodeling by the appearance of osteoclasts in opposition to the newly formed bone matrix. Vascular invasion is evident in these implants (FIGURE 3B). Formation is scored as (+++) by the presence of extensive remodeled bone which results in the formation of ossicles.

The overall bone inducing activity due to recombinant protein is represented as percent response of endochondral bone formation (see TABLE 2 below).

TABLE 2

HISTOLOGICAL EVALUATION OF RECOMBINANT BONE INDUCTIVE PROTEINS			
Sample No.	Implanted Protein	Cartilage Formation	Bone Formation
260-54	COP-5	+++	++
279-5	COP-5	++	+
285-13	COP-5	+++	++
277-7	COP-7	+++	++
277-8	COP-7	+++	++
277-9	COP-7	++	+
285-14	COP-7	+++	++
285-24	COP-7	++	+
285-25	COP-7	++	++
314-6	COP-16	+++	+++
314-15	COP-16	++	+
314-16	COP-16	++	+
314-12	OP-1	++	+

The present embodiments are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description.

Claims

1. Use of a composition for the manufacture of a medicament for inducing bone formation, the composition consisting essentially of dimeric osteogenic protein encoded by a single DNA sequence as the active osteogenic ingredient, said DNA sequence encoding a polypeptide chain comprising an amino acid sequence sufficiently duplicative of a sequence of

(COP5)

```
1       10          20          30          40
     LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
            50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(COP7)

```
1       10          20          30          40
     LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
            50          60          70
HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(COP16)

```
                          -10
                        PKHHSQRARKKNYKN
1       10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
            50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(OP1)

```
                                                        -5
                                                        HQRQA
1         10          20          30          40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
                50          60          70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH,
```

such that said polypeptide chain, when dimerized, is capable of inducing cartilage and endochondral bone formation when implanted in a mammal in association with a matrix.

2. The use according to claim 1, wherein said protein is obtainable by:

(a) constructing a DNA molecule comprising a DNA sequence of claim 1;

(b) introducing said DNA molecule into a host cell;

(c) culturing said host cell under conditions suitable for expressing said DNA molecule to produce a polypeptide chain; and

(d) recovering and purifying dimeric osteogenic proten comprising said polypeptide chain.

3. The use according to claim 1 or 2 wherein said osteogenic protein comprises the amino acid sequence:

(a) (CBMP2A)

```
1         10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; or
```

(b) (CBMP2B)

```
1         10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

4. The use according to any of the preceding claims wherein said polypeptide chain comprises a homologous or mutated form of the amino acid sequence:

(a) (CBMP2A)

```
1         10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
              50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; or
```

(b) (CBMP2B)

```
1         10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
              50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

5. The use according to any one of the preceding claims wherein said DNA sequence comprises cDNA.

6. The use according to any of claims 1-4 wherein said DNA sequence comprises genomic DNA or chemically synthesized oligonucleotides.

7. The use according to any of claims 2-6 wherein said host cell is a mammalian cell, e.g. a CHO cell.

8. The use according to any of claims 2-7 wherein said host cell is an E. coli, bacillus or yeast cell.

9. The use according to any one of the preceding claims wherein said polypeptide chain comprises less than 200 amino acids.

10. The use according to any one of the preceding claims wherein said medicament further comprises : (A) a matrix comprising: (a) allogenic bone, e.g. demineralized, protein extracted, particulate, allogenic bone, (b) demineralized, protein extracted, particulate, deglycosylated xenogenic bone, (c) demineralized, protein extracted, particulate xenogenic bone treated with HF or a protease, (d) materials selected from collagen, hydroxyapatite, calcium phosphates (e.g. tricalcium phosphate) and polymers comprising glycolic acid and/or lactic acid monomers, (e) a shape-retaining solid of loosely adhered particulate material e.g. collagen, (f) a porous solid or (g) masticated tissue, e. g. muscle; or (B) a carrier which acts as a slow release delivery system, accommodates each step of the cellular response during bone development, protects the osteogenic protein from non specific proteolysis and in biocompatible and biodegradable.

11. The use according to any of the preceding claims, said medicament being for inducing local bone formation in a mammal by implantation in a mammal at a locus accessible to migratory progenitor cells, for repairing non-union fractures, and for correcting acquired or congenital craniofacial and other skeletal or dental anomalies, including for periodontal treatment.

12. The use according to any one of the preceding claims, said medicament being for inducing local bone and cartilage formation.

13. An osteogenic delivery or support system adapted to induce bone formation in a mammal, comprising a composition as defined in claim 1 as the only active osteogenic ingredient, the delivery or support system being shaped to span a bone defect.

14. The osteogenic system of claim 13 wherein:

(A) the osteogenic protein is obtainable by the steps as defined in claim 2 (wherein for example the host cell is as defined in claim 7 or claim 8), or
(B) the osteogenic protein comprises; (a) the amino acid sequence of claim 3, or (b) the homologous or mutated form of the amino acid sequence as defined in claim 4; or
(C) the DNA sequence is as defined in claim 5 or claim 6;
(D) the polypeptide chain is as defined in claim 9; or
(E) the delivery or support system is a matrix or carrier as defined in claim 10; or
(F) the osteogenic delivery or support system is for the uses as defined in claim 11 or claim 12.

Patentansprüche

1. Verwendung einer Zusammensetzung für die Herstellung eines Medikaments zur Induktion der Knochenbildung, wobei die Zusammensetzung im wesentlichen aus einem dimeren osteogenen Protein, das von einer einzigen DNS-Sequenz codiert wird, als dem aktiven osteogenen Bestandteil besteht, wobei besagte DNS-Sequenz eine Polypeptidkette codiert, die eine Aminosäure-Sequenz umfaßt, die einer Sequenz

(Cop5)

```
1       10          20          30          40
     LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
            50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; oder
```

(COP7)

```
1       10          20          30          40
     LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
            50          60          70
HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; oder
```

(COP16)

```
                                -10
                             PKHHSQRARKKNYKN
1       10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
            50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; oder
```

```
(OP1)
                                          -5
                                          HQRQA
1       10          20          30          40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
            50          60          70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
    80          90          100
ISVLYFDDSSNVILKKYRNMVVRACGCH;
```

hinreichend entspricht, so daß besagte Polypeptidkette, wenn dimerisiert, in der Lage ist, die Knorpel- und endochondrale Knochenbildung zu induzieren, wenn sie in einen Säuger in Verbindung mit einer Matrix implantiert ist.

2. Die Verwendung gemäß Anspruch 1, worin besagtes Protein erhältlich ist durch:

   (a) Konstruieren eines DNS-Moleküls, das eine DNS-Sequenz gemäß Anspruch 1 umfaßt;

   (b) Einführen besagten DNS-Moleküls in eine Wirtszelle;

   (c) Kultivieren besagter Wirtszelle unter für eine Expression besagten DNS-Moleküls geeigneten Bedingungen, um eine Polypeptidkette herzustellen; und

   (d) Wiedergewinnen und Reinigen des dimeren osteogenen Proteins, das besagte Polpeptidkette umfaßt.

3. Die Verwendung gemäß Anspruch 1 oder 2, worin besagtes osteogenes Protein die Aminosäure-Sequenz umfaßt:

```
(a) (CBMP2A)

1       10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
            50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
    80          90          100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; oder

(b) (CBMP2B)

1       10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
            50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
    80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

4. Die Verwendung gemäß einem der vorhergehenden Ansprüche, worin besagte Polypeptidkette eine homologe oder mutierte Form der Aminosäure-Sequenz umfaßt:

(a) (CBMP2A)

```
1        10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
             50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; oder
```

(b) (CBMP2B)

```
1        10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
             50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90          100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

5. Die Verwendung gemäß einem der vorhergehenden Ansprüche, worin besagte DNS-Sequenz cDNS umfaßt.

6. Die Verwendung gemäß einem der Ansprüche 1 bis 4, worin besagte DNS-Sequenz genomische DNS oder chemisch synthetisierte Oligonucleotide umfaßt.

7. Die Verwendung gemäß einem der Ansprüche 2 bis 6, worin besagte Wirtszelle eine Säugerzelle, z. B. eine CHO-Zelle, ist.

8. Die Verwendung gemäß einem der Ansprüche 2 bis 7, worin besagte Wirtszelle eine E. coli, Bacillus oder Hefezelle ist.

9. Die Verwendung gemäß einem der vorhergehenden Ansprüche, worin besagte Polypeptidkette weniger als 200 Aminosäuren umfaßt.

10. Die Verwendung gemäß einem der vorhergehenden Ansprüche, worin besagtes Medikament ferner umfaßt: (A) eine Matrix, die umfaßt: (a) allogenen Knochen, z. B. demineralisierten, Protein extrahierten, partikulären, allogenen Knochen, (b) demineralisierten, Protein extrahierten, partikulären, deglycosylierten xenogenen Knochen, (c) demineralisierten, Protein extrahierten, partikulären xenogenen Knochen, behandelt mit HF oder einer Protease, (d) Materialien, gewählt aus Kollagen, Hydroxyapatit, Calciumphosphaten (z. B. Tricalciumphosphat) und Polymeren, die Glycolsäure und/oder Milchsäure Monomere umfassen, (e) einen formstabilen Festkörper aus locker haftendem partikulären Material z. B. Kollagen, (f) einen porösen Festkörper oder (g) zerkleinertes Gewebe z. B. Muskel; oder (B) einen Träger, der als ein langsames Abgabe-Liefer-System wirkt, jeden Schritt der Zellantwort während der Knochenentwicklung versorgt, das osteogene Protein vor unspezifischer Proteolyse schützt und biokompatibel und biologisch abbaubar ist.

11. Die Verwendung gemäß einem der vorhergehenden Ansprüche, wobei besagtes Medikament zur Induktion lokaler Knochenbildung in einem Säuger durch Implantation in einen Säuger an einen Ort, der für migrierende vorläuferzellen zugänglich ist, zur Wiederherstellung nicht zusammenwachsender Frakturen und zur Korrektur erworbener oder kongenitaler craniofacialer und anderen Skelettanomalien oder dentalen Anomalien, einschließlich zur periodontalen Behandlung, dient.

12. Die Verwendung gemäß einem der vorhergehenden Ansprüche, wobei besagtes Medikament zur Induktion lokaler Knochen- und Knorpelbildung dient.

13. Ein osteogenes Liefer- oder Versorgungssystem, das adaptiert ist, Knochenbildung in einem Säuger zu induzieren, das eine Zusammensetzung, wie in Anspruch 1 definiert, als den einzigen aktiven osteogenen Bestandteil umfaßt, wobei das Liefer- oder Versorgungssystem geformt ist, um einen Knochendefekt zu überbrücken.

14. Das osteogene System nach Anspruch 13, worin:

(A) das osteogene Protein durch die Schritte wie in Anspruch 2 definiert (worin beispielsweise die Wirtszelle, wie in Anspruch 7 oder Anspruch 8 definiert, ist) erhältlich ist, oder
(B) das osteogene Protein umfaßt; (a) die Aminosäure-Sequenz nach Anspruch 3, oder (b) die homologe oder mutierte Form der Aminosäure-Sequenz, wie in Anspruch 4 definiert;
oder
(C) die DNS-Sequenz so ist, wie in Anspruch 5 oder Anspruch 6 definiert;
(D) die Polypeptidkette so ist, wie in Anspruch 9 definiert; oder
(E) das Liefer- oder Versorgungssystem eine Matrix oder ein Träger ist, wie in Anspruch 10 definiert; oder
(F) das osteogene Liefer- oder Versorgungssystem für die Verwendungen dient, wie in Anspruch 11 oder Anspruch 12 definiert.

Revendications

1. Utilisation d'une composition pour la fabrication d'un médicament pour l'induction d'une formation osseuse, ladite composition consistant essentiellement en une protéine ostéogénique dimérique codée par une séquence d'ADN unique en tant qu'ingrédient ostéogénique actif, ladite séquence d'ADN codant pour une chaîne polypeptidique comprenant une séquence d'acides aminés suffisamment duplicative de la séquence de

(COP5)

```
1          10          20          30          40
      LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
              50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(COP7)

```
1          10          20          30          40
      LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
              50          60          70
HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
     80          90          100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(COP16)

```
                                    -10
                                  PKGHSQRARKKNYKN
1         10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
               50          60          70
HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
     80          90         100
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or
```

(CP1)

```
                                                  -5
                                                HQRQA
1         10          20          30          40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
               50          60          70
HMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
     80          90         100
ISVLYFDDSSNVILKKYRNMVVRACGCH.
```

de telle sorte que ladite chaîne polypeptidique, lorsqu'elle est dimérisée, est capable d'induire la formation de cartilage et d'os endochondral lorsqu'elle est implantée chez un mammifère en association avec une matrice.

2. Utilisation selon la revendication 1, où ladite protéine peut être obtenue par :

   (a) construction d'une molécule d'ADN comprenant une séquence d'ADN de la revendication 1 ;
   (b) introduction de ladite molécule d'ADN dans une cellule hôte ;
   (c) culture de ladite cellule hôte dans des conditions appropriées pour l'expression de ladite molécule d'ADN pour produire une chaîne polypeptidique; et
   (d) récupération et purification de la protéine ostéogénique dimérique comprenant ladite chaîne polypeptidique.

3. Utilisation selon la revendication 1 ou 2, où ladite protéine ostéogénique comprend la séquence d'acides aminés :

(a) (CBMP2A)

```
1         10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
     80          90         100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; or
```

(b) (CBMP2B)

```
1         10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90         100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

4. Utilisation selon l'une quelconque des revendications précédentes, où ladite chaîne polypeptidique comprend une forme homologue ou mutée de la séquence d'acides aminés :

(a) (CBMP2A)

```
1         10          20          30          40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
     80          90         100
ISMLYLDENEKVVLKNYQDMVVEGCGCR; or
```

(b) (CBMP2B)

```
1         10          20          30          40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
               50          60          70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
     80          90         100
ISMLYLDEYDKVVLKNYQEMVVEGCGCR.
```

5. Utilisation selon l'une quelconque des revendications précédentes, où ladite séquence d'ADN comprend un ADNc.

6. Utilisation selon l'une quelconque des revendications 1 à 4, où ladite séquence d'ADN comprend de l'ADN génomique ou des oligonucléotides synthétisés chimiquement.

7. Utilisation selon l'une quelconque des revendications 2 à 6, où ladite cellule hôte est une cellule de mammifère, par exemple une cellule CHO.

8. Utilisation selon l'une quelconque des revendications 2 à 7, où ladite cellule hôte est une cellule de E. Coli, de bacillus ou de levure.

9. Utilisation selon l'une quelconque des revendications précédentes, où ladite chaîne polypeptidique comprend moins de 200 acides aminés.

10. Utilisation selon l'une quelconque des revendications précédentes, où ledit médicament comprend en outre :

(A) une matrice comprenant :

(a) de l'os allogénique, par exemple de l'os allogénique, déminéralisé, exempt de protéine, sous forme de particules,
(b) de l'os xénogénique, déglycosylé, déminéralisé, exempt de protéine, sous forme de particules,
(c) de l'os xénogénique déminéralisé exempt de protéine sous forme de particules, traité par du fluorure d'hydrogène ou une protéase,
(d) un matériau choisi parmi le collagène, l'hydroxyapatite, les phosphates de calcium (ex. : phosphate tricalcique) et des polymères comprenant des monomères d'acide glycolique et/ou lactique,
(e) un solide pour maintenir la forme, en une matière composée de particules faiblement adhérées ex. le collagène,
(f) un solide poreux, ou
(g) un tissu mastiqué, ex. : le muscle ; ou

(B) un véhicule qui agit comme un système de délivrance à libération lente, subvient à chaque étape de la réponse cellulaire durant le développement de l'os, protège la protéine ostéogénique d'une protéolyse non spécifique, et est biocompatible et biodégradable.

11. Utilisation selon l'une quelconque des revendications précédentes, ledit médicament étant destiné à l'induction d'une formation osseuse locale chez un mammifère par implantation chez un mammifère à un lieu accessible aux cellules progénitrices migratoires, pour réparer des fractures non-jointives et pour corriger les anomalies crano-faciales ou autres anomalies squelettiques ou dentaires acquises ou congénitales, y compris pour un traitement périodontal.

12. Utilisation selon l'une quelconque des revendications précédentes, ledit médicament étant destiné à l'induction d'une formation d'os et de cartilage locale.

13. Système de délivrance ostéogénique ou système de support adapté à l'induction d'une formation osseuse chez un mammifère, comprenant une composition telle que définie dans la revendication 1 ayant comme seul ingrédient ostéogénique actif, le système de délivrance ou de support dont la forme recouvre un défaut osseux.

14. Système ostéogénique selon la revendication 13, où :

(A) la protéine ostéogénique peut être obtenue par les étapes telles que définies dans la revendication 2 (où par exemple la cellule hôte est telle que définie dans la revendication 7 ou 8), ou
(B) la protéine ostéogénique comprend :

(a) la séquence d'acides aminés de la revendication 3, ou
(b) la forme homologue ou mutée de la séquence d'acides aminés, telle que définie dans la revendication 4 ; ou

(C) la séquence d'ADN est telle que définie dans la revendication 5 ou 6 ;
(D) la chaîne polypeptidique est telle que définie dans la revendication 9 ; ou
(E) le système de délivrance ou de support est une matrice ou un véhicule tel que défini dans la revendication 10 ;
(F) le système de délivrance ou support ostéogénique est destiné à des utilisations telles que définies dans la revendication 11 ou 12.

FIG. 1-1

COP1 COP5 / COP3 COP7 / COP4	VG1	DPP	OP1	CBMP2 a)	CBMP2 b)	CBMP3 (bov)	beta-Inhibin a)	beta-Inhibin b)	TGF-beta 1 2 3	MIS	alpha-Inhib	consensus choices
	C	C	C	C		C	C	C	C	C	C	c
	K	R	K	K	R	A	C	C	C	A	H	k,r
<P P P P P>	K	R	K	R		R	K	R	V L V	L	R	k,r
<N N N N N>	R	H	H	H		R	K	Q	R	R	V	h,k,r,q
<G G G G G>	H	S	E	P	S	Y	Q	Q	Q P P	E	A	p,s,e,q
L L L L L	L	L	L	L		L	F	F	L	L	L	l
Y Y Y Y Y	Y	Y	Y	Y		K	F	F	Y	S	N	y,f
V V V V V	V	V	V	V		V	V	I	I	V	I	v,i
D D D D D	E	D	S	D		D	S	D	D	D	S	d,e,s
F F F F F	F	F	F	F		F	F	F	F	L	F	f
Q Q S S S	K	S	R	S		A	K	R	R K R	R	Q	k,r,s
R R									K R Q	A		
D D D D D	D	D	D	D		D	D		D	E	E	d
V V V V V	V	V	L	V		I	I	I	L	R	L	v,l,i
G G G G G	G	G	G	G		G	G	G	G	S	G	g
W W W W W	W	W	W	W		W	W	W	W		W	w
D D D D N	Q	D	Q	N		S	N	N	K		E	q,n,d,e,s
D D D D D	N	D	D	D		E	D	D	-		R	d,e,n
W W W W W	W	W	W	W		W	W	W	W		W	w
I I I I I	V	I	I	I		I	I	I	I I V	V	I	i,v
I V V V V	I	V	I	V		I	I	I	H	L	V	i,v
A A A A A	A	A	A	A		S	A	A	E	I	Y	a,s
P P P P P	P	P	P	P		P	P	P	P	P	P	p
V P P P P	Q	L	E	P		K	S	T	K	E	P	e,q,p*
D G G G G	G	G	G	G		S	G	G	G	T	S	g,s
F Y Y Y Y	Y	Y	Y	Y		F	Y	Y	Y	Y	F	y,f
D Q Q Q H	M	D	V	H		D	H	Y	H N Y	Q	I	h,d,n,q,y*
A A A A A	A	A	A	A		A	A	G	A	A	F	a

EP 0 372 031 B1

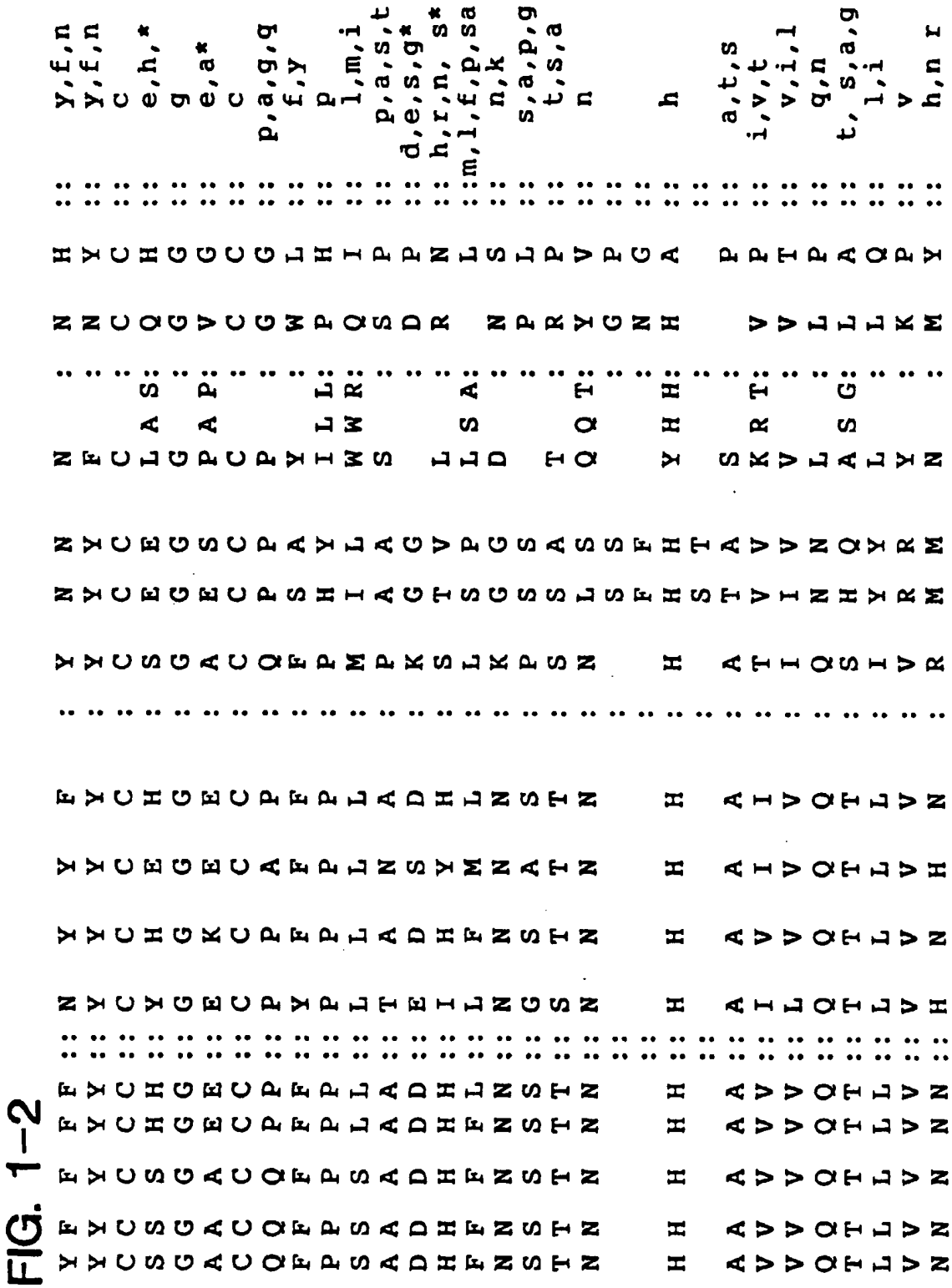


FIG. 1–2

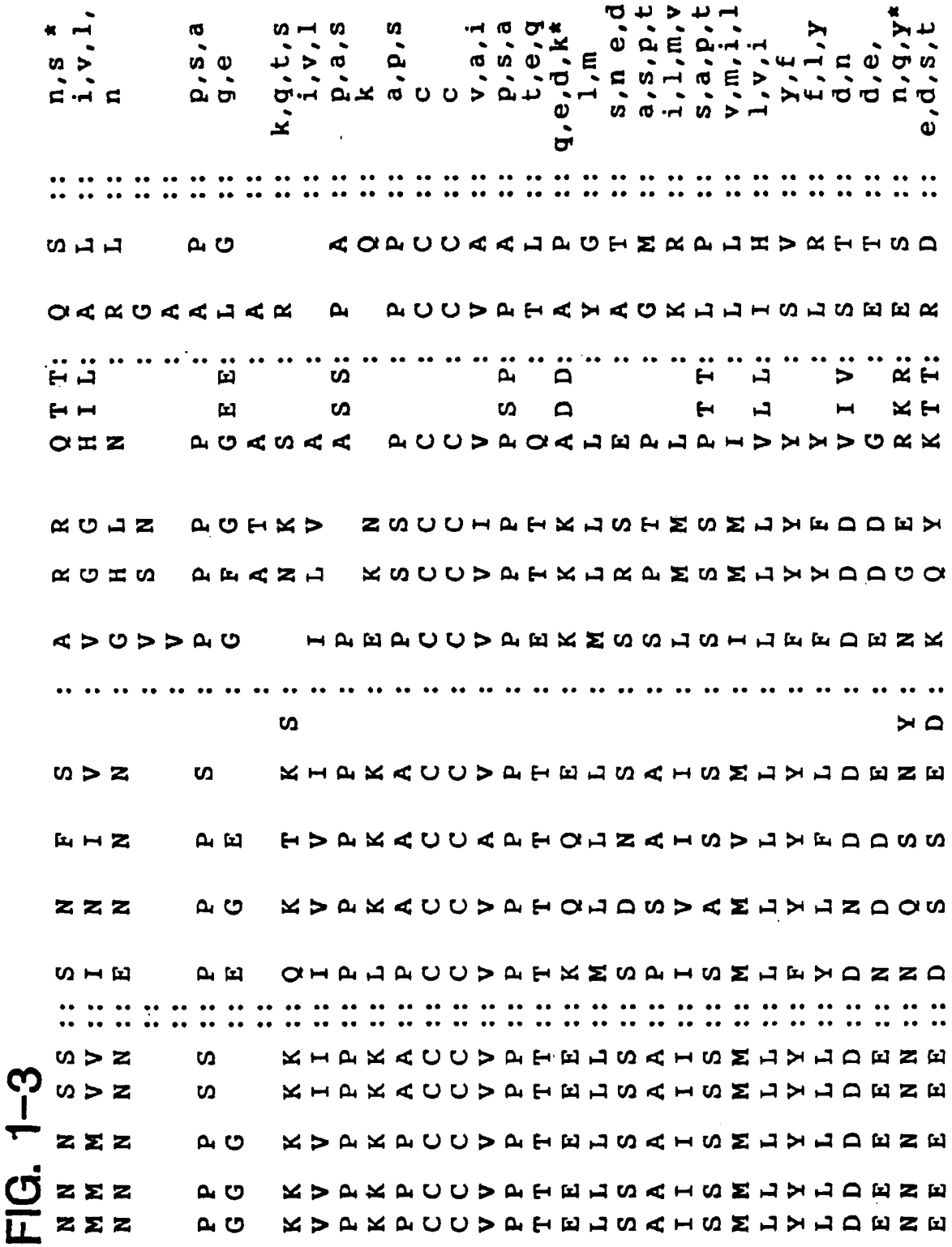


FIG. 1–3

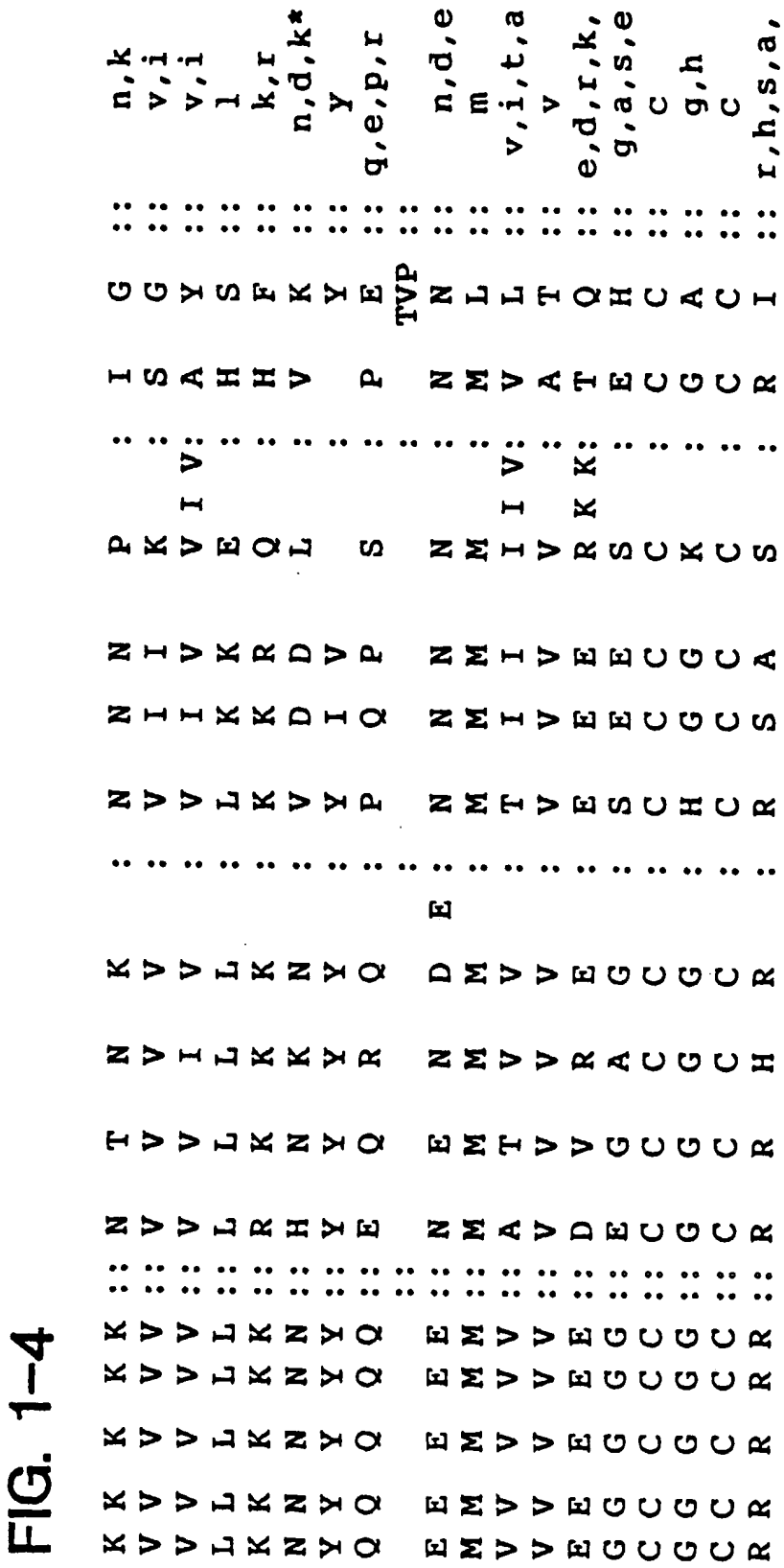
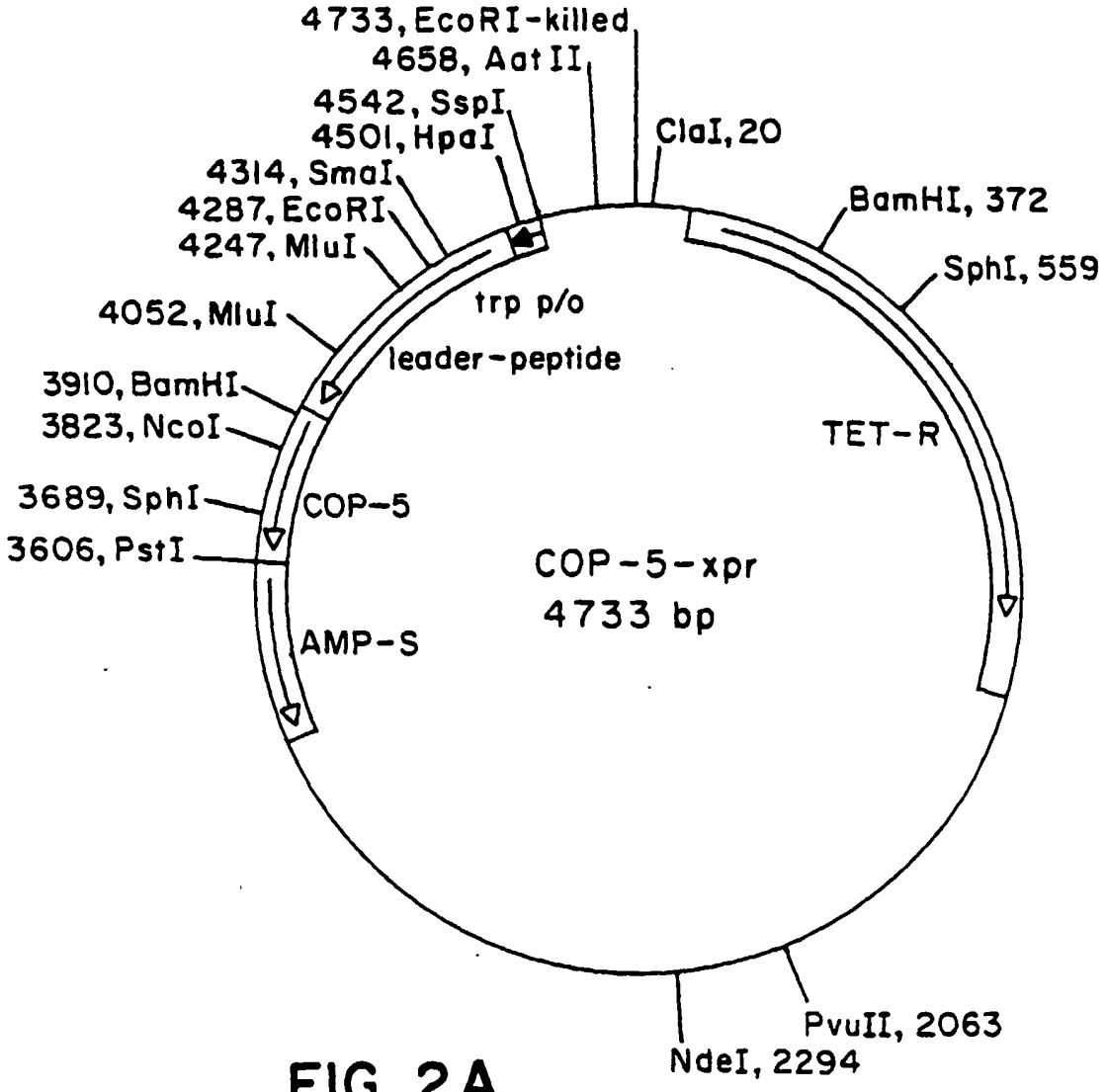


FIG. 1–4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4658, AatII
4542, SspI
4501, HpaI
4314, SmaI
4287, EcoRI
4247, MluI
4052, MluI
3910, BamHI
3823, NcoI
3689, SphI
3606, PstI
ClaI, 20
BamHI, 372
SphI, 559
trp p/o
leader-peptide
TET-R
COP-5
COP-5-xpr
4733 bp
AMP-S
PvuII, 2063
NdeI, 2294

FIG. 2A

FIG. 2B-1

COP-5 fusion protein

```
        10         20         30         40         50
ATGAAAGCAATTTTCGTACTGAAAGGTTCACTGGACAGAGATCTGGACTC
 M  K  A  I  F  V  L  K  G  S  L  D  R  D  L  D  S
                                          BglII

        60         70         80         90        100
TCGTCTGGATCTGGACGTTCGTACCGACCACAAAGACCTGTCTGATCACC
  R  L  D  L  D  V  R  T  D  H  K  D  L  S  D  H

       110        120        130        140        150
TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCC
L  V  L  V  D  L  A  R  N  D  L  A  R  I  V  T  P
        SalI                                      Sma

       160        170        180        190        200
GGGTCTCGTTACGTTGCGGATCTGGAATTCATGGCTGACAACAAATTCAA
 G  S  R  Y  V  A  D  L  E  F  M  A  D  N  K  F  N
I                       EcoRI

       210        220        230        240        250
CAAGGAACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGA
  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L
             MluI        BglII    BspMI+

       260        270        280        290        300
ACGAAGAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCT
N  E  E  Q  R  N  G  F  I  Q  S  L  K  D  E  P  S
                            HindIII

       310        320        330        340        350
CAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGC
 Q  S  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A
             NheI                            FspI

       360        370        380        390        400
ACCGAAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGG
  P  K  S  D  Q  G  Q  F  M  A  D  N  K  F  N  K

       410        420        430        440        450
AACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGAACGAA
E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L  N  E
        MluI        BglII    BspMI+

       460        470        480        490        500
GAGCAGCGTAACGGCTTCATCCAAAGCTTGAAGGATGAGCCCTCTCAGTC
 E  Q  R  N  G  F  I  Q  S  L  K  D  E  P  S  Q  S
                      HindIII
```

FIG. 2B-2

```
       510        520        530        540        550
TGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCGCAGGCACCGA
  A  N  L  L  A  D  A  K  K  L  N  D  A  Q  A  P
         NheI                              FspI

       560        570        580        590        600
AGGATCCTAATGGGCTGTACGTCGACTTCAGCGACGTGGGCTGGGACGAC
K  D  P  N  G  L  Y  V  D  F  S  D  V  G  W  D  D
 BamHI              SalI

       610        620        630        640        650
TGGATTGTGGCCCCACCAGGCTACCAGGCCTTCTACTGCCATGGCGAATG
 W  I  V  A  P  P  G  Y  Q  A  F  Y  C  H  G  E  C
                           StuI          NcoI   BsmI+

       660        670        680        690        700
CCCTTTCCCGCTAGCGGATCACTTCAACAGCACCAACCACGCCGTGGTGC
  P  F  P  L  A  D  H  F  N  S  T  N  H  A  V  V
         NheI                              DraIII
                                           PflMI

       710        720        730        740        750
AGACCCTGGTGAACTCTGTCAACTCCAAGATCCCTAAGGCTTGCTGCGTG
Q  T  L  V  N  S  V  N  S  K  I  P  K  A  C  C  V
                                    MstII

       760        770        780        790        800
CCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATGAGAA
 P  T  E  L  S  A  I  S  M  L  Y  L  D  E  N  E  K
                        SphI

       810        820        830        840        850
GGTGGTGCTGAAGAACTACCAGGAGATGGTAGTAGAGGGCTGCGGCTGCC
  V  V  L  K  N  Y  Q  E  M  V  V  E  G  C  G  C
                     PflMI

       860
GCTAACTGCAG
R  *
     PstI
```

FIG. 3A

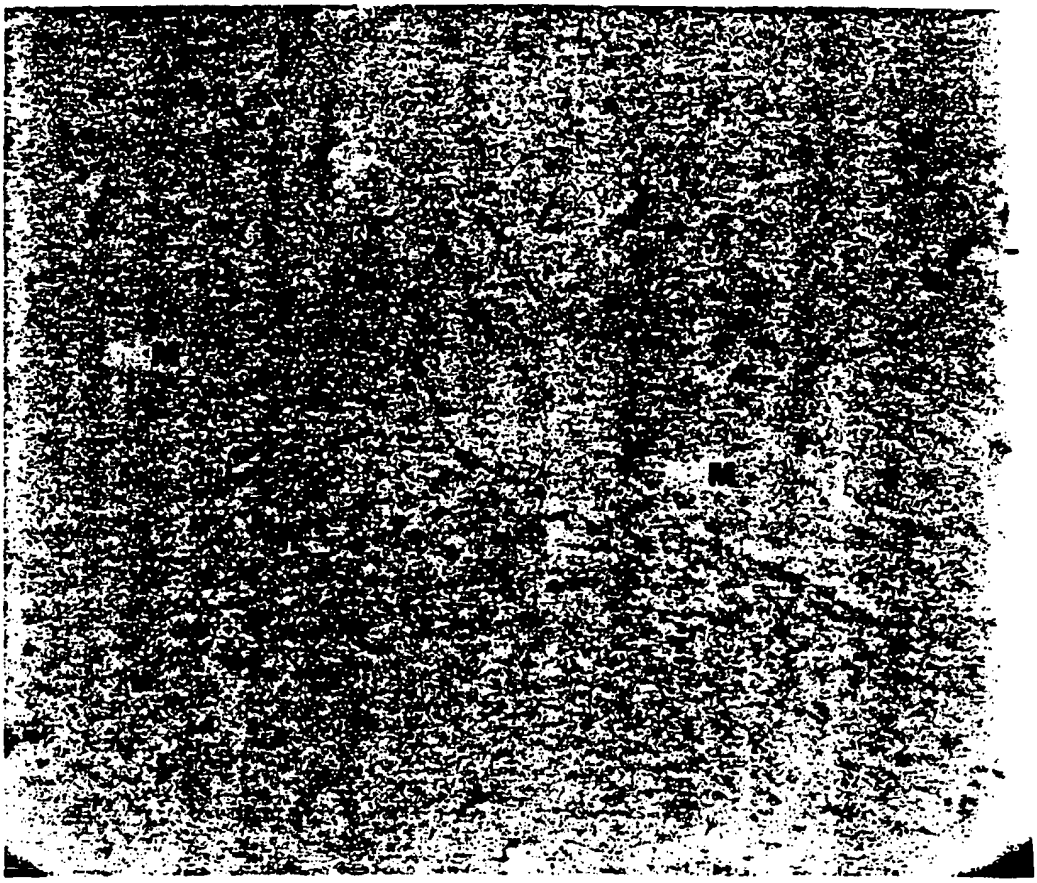
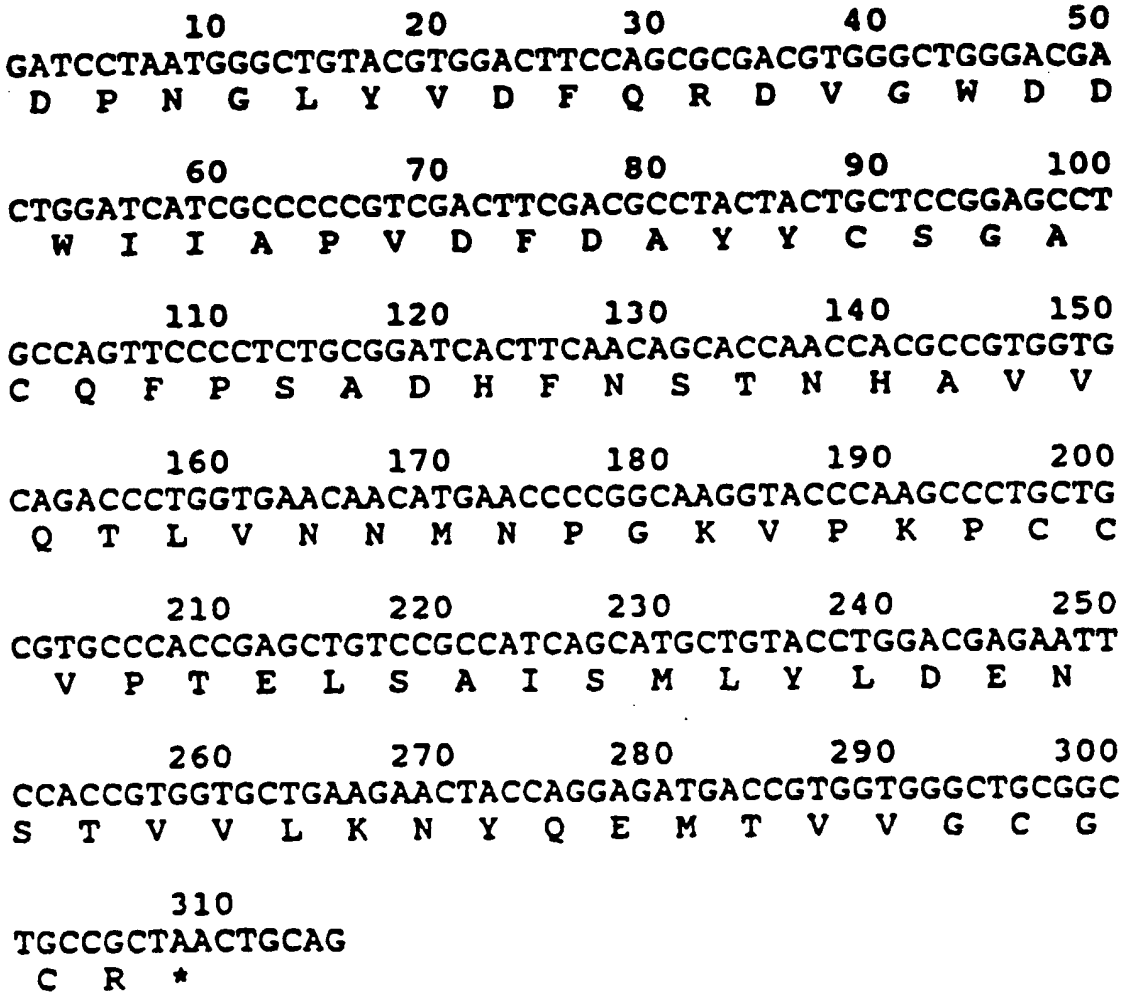


FIG. 3B

FIG. 4


10 20 30 40 50
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGA
D P N G L Y V D F Q R D V G W D D
60 70 80 90 100
CTGGATCATCGCCCCCGTCGACTTCGACGCCTACTACTGCTCCGGAGCCT
W I I A P V D F D A Y Y C S G A
110 120 130 140 150
GCCAGTTCCCCTCTGCGGATCACTTCAACAGCACCAACCACGCCGTGGTG
C Q F P S A D H F N S T N H A V V
160 170 180 190 200
CAGACCCTGGTGAACAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTG
Q T L V N N M N P G K V P K P C C
210 220 230 240 250
CGTGCCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATT
V P T E L S A I S M L Y L D E N
260 270 280 290 300
CCACCGTGGTGCTGAAGAACTACCAGGAGATGACCGTGGTGGGCTGCGGC
S T V V L K N Y Q E M T V V G C G
310
TGCCGCTAACTGCAG
C R *